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(54) Title: MUTANTS OF HIV FOR SUPRESSION OF HIV INFECTION

(57) Abstract

The invention features compositions and methods useful for the treatment of HIV-infected individuals and for patients with AIDS.

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MUTANTS OF HIV FOR SUPPRESSION OF HIV INFECTION

BACKGROUND OF THE INVENTION

The invention relates to compositions and methods for the treatment of Acquired Immunodeficiency Syndrome.

Human Immunodeficiency Virus (HIV) is the etiologic agent of a fatal immunodeficiency condition in humans termed Acquired Immunodeficiency Syndrome or AIDS. As far as is known to date, all patients infected with HIV eventually progress to AIDS Related Complex (ARC), then AIDS and subsequent death. HIV-infected individuals may remain clinically healthy for prolonged periods of time, with an estimated average incubation period of ten years for adults and less for children.

HIV is a retrovirus which primarily infects cells of the immune system, although involvement of other cells is documented. Infection of cells with HIV is characterized by cycles of active gene expression and relative latency, which cycles are controlled in large part at the level of transcription. The initial transcriptional activation of the HIV genome during primary infection and emergence from the latent state is dependent upon the interaction of host cell transcription factors with *cis*-acting elements in the viral long terminal repeat (LTR) region. Experimental studies using a systematic mutagenesis approach have delineated several *cis*-acting elements in the LTR and have implicated specific DNA-binding proteins in the regulation of transcription of the HIV genome (Gaynor, *AIDS* **(1992)** 6:347). This has been accomplished using transient transfection assays (Harrich et al., *J. Virol.* **(1989)** 63:2585; Muesing et al., *Cell* **(1987)**

48:691; Rosen et al., *Cell* **(1985)** 41:813; Zeichner et al., *J. Virol.* **(1991)** 65:2436-2444) and in studies in which the biological properties of viruses with discrete mutations were examined in order to analyze the influence of cellular factors in the context of the viral life cycle (Harrich et al., *EMBO J.* **(1990)** 9:4417; Lu et al., *J. Virol.* **(1989)** 63:4115).

Currently, there is no cure for AIDS. There is an acute need for the development of therapies which may be used either alone or in combination with existing therapies for the treatment of AIDS. These therapies could be useful in management of the disease and in particular for retarding the progression of a clinically healthy HIV-infected individual to ARC and AIDS.

SUMMARY OF THE INVENTION

The invention features conditional replication incompetent (CRI) mutants of HIV which have the potential to become replication competent when the virus-specific Tat protein is supplied in trans, for example, by an incoming wild type (replication-competent) HIV. The mutants of the invention encode a replication-incompetent LTR region whose activation is stringently dependent upon the expression of the Tat protein supplied in trans.

Preferably, the CRI mutants of the invention encode mutations which are linker substitution mutations, although other substitution, insertion, deletion or point mutations are also included in the invention.

The mutations in the genome of the mutant viruses of the invention are positioned between nucleotides -453 to -57 relative to the +1 transcriptional start site on the HIV proviral DNA. Preferably, the mutations are positioned between nucleotides -453 to -111, and more preferably, the mutations are positioned between nucleotides -435 to -111, relative to the transcriptional start site. Even more preferably, mutations are positioned between nucleotides -201 to -130, relative to the transcriptional start site. Most preferably, the mutant viruses of the invention are mutants of HIV type 1

(HIV-1) selected from the group consisting of -147/-130 NXS, -165/-148 NXS or -201/-184 NXS. The mutant viruses of the invention are conditionally replication incompetent in cells of the immune system, such as peripheral blood lymphocytes, or cells established from immune cells, such as 11.8 cells, or in cells of the nervous system.

By conditional replication incompetent HIV is meant a virus which can maintain residence in a cell but cannot replicate unless a wild type form of the HIV-specific Tat gene, encoded by an HIV proviral DNA other than the proviral DNA of the conditional replication incompetent virus, is expressed in that cell.

The term "HIV" as used herein, includes both HIV-1 and HIV type 2 (HIV-2).

By the term "Tat supplied in trans", as used herein, is meant the expression of Tat encoded by a proviral DNA other than the proviral DNA of the mutant virus. The Tat gene may be encoded by HIV-1, HIV-2, a transiently or stably transfected expression vector such as a plasmid, an integrated retroviral expression vector, or any other wild type strain of HIV, since Tat so expressed from one strain of HIV is capable of activating other strains of HIV.

The invention also features CRI mutants of HIV such as those described above, wherein these mutants further encode a gene product (either RNA or protein) capable of either inhibiting replication of wild type HIV or of killing a cell in which the mutant resides. The gene for this additional protein is positioned in the mutated HIV genome such that expression of the gene is stringently controlled by the LTR region. A unique property of these mutants is their replication-incompetence in the absence of exogenously supplied Tat protein and their potential to be activated to a productive mode of gene expression in the presence of Tat protein expressed in trans from a wild type HIV proviral DNA. Thus, the inhibitory gene is only expressed in cells which are, or become, infected with a replication-competent HIV.

The inhibitory gene encoded by the mutants of the invention is a gene encoding a transdominant form of an HIV protein such as Rev, Gag or Tat, or it is a gene encoding a transdominant form of a gene encoded by a heterologous virus such as the Tax or Rex gene of Human T cell leukemia virus type 1 (HTLV-1). The inhibitory gene is also a gene encoding an antisense RNA sequence capable of inhibiting replication of HIV, or it is a gene encoding a ribozyme also capable of inhibiting replication of HIV. The inhibitory gene encoded by the mutants of the invention is also a gene capable of killing a cell, such as a toxin, and preferably, a toxin such as the Ricin A subunit.

The viral mutants of the invention may also encode more than one inhibitory gene. For example, the CRI mutants may encode both a transdominant viral protein and an inhibitory antisense RNA sequence.

By transdominant form of a gene is meant a gene encoding a product with a dominant negative phenotype over the wild type function of the same or a similar gene, i.e., the product is capable of preventing other wild type forms of the same or a similar gene from functioning properly.

Mutant viruses according to the invention are viruses which are lymphotropic and/or monocyte/macrophage tropic and these viruses also optionally encode additional mutations which render the viruses replication-incompetent in cells of the nervous system, such as glial cells.

The CRI mutants of the invention are useful as therapeutic agents for treatment of HIV-infected individuals, and for treatment of patients with AIDS. When such mutants are introduced into cells which are themselves latently or actively infected with wild-type HIV, or which will become infected with HIV, they have the potential to remain replication-incompetent in the absence of exogenously supplied Tat. However, if the resident wild-type virus is in, or enters, an active replication phase, as is the case when the virus reactivates from the latent state or when the mutant infected cell becomes infected with wild type HIV, then Tat will be expressed in

these cells. Expression of Tat will activate the mutated LTR which in turn will drive expression of an inhibitory gene product encoded by the mutant genome. Expression of this gene will subsequently effect inhibition of replication of both wild-type and mutant viruses, or will effect death of the infected cell. Likewise, the introduction of the replication-incompetent mutants of the invention into uninfected cells of an HIV-positive patient should result in cells which are ostensibly normal. However, if these cells become infected with wild type HIV during the course of the patient's disease, the Tat protein will be expressed from the wild type genome resulting in activation of the mutated LTR, which will in turn, drive expression of the inhibitory gene product. The expression of the inhibitory gene product will subsequently effect inhibition of replication of both the wild type and mutant viruses, or will effect death of the infected cell. In either case, this strategy can be used to retard the spread of wild type HIV in an individual, and to retard progression of asymptomatic HIV-infected individuals to ARC and AIDS. It may also be useful in reducing the severity of the disease in patients who have already progressed to AIDS.

Thus, the invention also features methods of treating a patient infected with HIV. One such method involves administering the CRI mutant virus of the invention to the patient by injecting the virus directly into the patient's blood stream using a syringe or any other intravenous delivery system. In another method of the invention, the CRI mutant virus is administered to the patient by infecting, *in vitro*, T cells and/or monocytes which have been isolated from the blood of the patient, and then returning the infected cells to the blood stream of the patient. In yet another method of the invention, the CRI mutant virus is administered to the patient by infecting, *in vitro*, bone marrow progenitor stem cells which have been obtained from the patient, and then returning the infected cells to the bone marrow following infection.

The therapeutic methods of the invention have a significant advantage over other proposed gene therapy-type

protocols for treatment of HIV-infected patients because these proposed protocols all involve the introduction of a gene encoding an anti-HIV product into normal or HIV-infected cells, which gene is constitutively expressed in those cells. In the instant invention, a gene capable of inhibiting replication of HIV, or of killing an HIV-infected cell, is introduced into a cell under the control of an inducible promoter. Thus, this gene is not constitutively expressed in these cells; rather, it is only expressed when its particular effect is desired. Therefore, the cells in which the inhibitory gene resides are free of any potential negative side-effects which may result from the constitutive expression of that gene which is not normally constitutively expressed in those cells.

The CRI mutants of the invention may also be useful as a vaccine which when inoculated into HIV-negative individuals may serve to protect such individuals from subsequent infection with HIV.

Other features and advantages of the invention will be apparent from the following description of preferred embodiments thereof, and from the claims.

DETAILED DESCRIPTION

The drawings will first be briefly described.

The Drawings

Figure 1 is a map of HIV-1 LTR DNA illustrating the relative positions of transcription factor binding sites and a set of NXS linker substitution mutants. Abbreviations are as follows: AP1, activator protein 1; NRE, negative regulatory element; IL2/IL2R, interleukin-2/interleukin-2 receptor binding site; NFAT, nuclear factor of activated T-cells; USF 1, upstream stimulatory factor 1; $NF_{\kappa}B$, nuclear factor $_{\kappa}B$; TA, TATA element; I, initiator element; TAR, trans-acting responsive element; LBP/UBP, leader or untranslated binding protein. Scale refers to nucleotide position relative to the transcription start site (+1).

Figure 2 is similar to Figure 1 and is a map of HIV-1 LTR DNA illustrating the relative positions of transcription factor binding sites and linker-substitution mutations which have been shown, thus far, to confer conditional replication incompetence. In the left column, CAT activity values represent averages relative to wild-type LTR activity in multiple transient transfections of PHA/PMA-stimulated Jurkat cells. In the expanded view at the bottom, open boxes represent wild-type sequences and filled boxes represent NXS linker-substitutions. Abbreviations are as follows: AP1, activator protein 1; NRE, negative regulatory element; IL2/IL2R, interleukin-2/interleukin-2 receptor binding site; NFAT, nuclear factor of activated T-cells; URS, upstream repression sequence; USF 1, upstream stimulatory factor 1; TCF-1α, T-cell factor 1α/lymphoid enhancer binding factor 1; $NF_{\kappa}B$, nuclear factor $_{\kappa}B$; TA, TATA element; I, initiator element; TAR, trans-acting responsive element; LBP/UBP, leader or untranslated binding protein. Scale refers to nucleotide position relative to the transcription start site (+1).

Figure 3 illustrates the construction of linker-substitution mutant LTR-containing proviruses. The figure depicts the relevant viral elements in the background of the appropriate plasmids. (A) The wild-type, terminally repetitious NL4-3 proviral fragment with a single LTR (open rectangle) and an intact *Xho*I site obtained by *BamH*I digestion of pILIC. (B) pG3-ILICX, illustrating the mutation in the *Xho*I site by insertion of an *Xba*I linker containing stop codons in each reading frame of *nef*. (C) pG3-ILICX was digested with *Xba*I and *Sph*I to release the *Xba*I-*Sph*I LTR-containing fragment which was then cloned into pG3H-LTR for exchange of LTRs to introduce linker substitution mutations. (D) The *Xba*I-*Hind*III LTR fragment from linker-substitution mutant plasmids, illustrating the NXS linker (filled box). (E) The *Xba*I-*Hind*III LTR fragment of (D) which has replaced corresponding wild-type LTR sequences in pG3H-LTR (C) to generate the pG3H(*n/m*)NXS series. (F) The *Xba*I-*Sph*I fragment of pG3H(*n/m*)NXS which has replaced the wild-type LTR fragment in pG3-ILICX (B) to

generate the pG3I(*n/m*)NXS series. (G) For transfection/cocultivation experiments, concatamers of *BamHI* proviral fragments of pG3I(*n/m*)NXS (as shown) and pG3IWT (wild-type; a pG3-ILICX construct containing the wild type LTR sequences derived from the HXB2 strain of HIV-1, which was also the parent strain in the construction of the NXS linker scanning mutant LTRs) were generated, which restored LTRs to appropriate 5'- and 3'-positions relative to NL4-3 coding sequences. Scale at the bottom is in kilobases. Abbreviations are as follows: B, *BamHI*; H, *Hind*III; S, *Sph*I; kb, kilobases.

Figure 4A, B and C are graphs depicting replication of wild-type HIV-1 and NXS linker-substitution mutant HIV-1 viruses in human peripheral blood lymphocytes (PBLs) and T cell lines. Equal aliquots of filtered and standardized virus stocks from RD/CEMx174 cocultures were used to inoculate 1 X 10^6 cells. Virus replication was measured using $p24^{gag}$ ELISA. Each data point represents the mean of duplicate infections. (A) Infection of PBLs stimulated with PHA, then maintained in IL-2. (B) Infection of 11.8 cells. (C) Infection of CEMx174 cells.

There will now be described the experimental details for the practice of the invention. First, the generation and characterization of replication-incompetent mutants will be described; second, there will be described the methods for construction and propagation of replication-incompetent HIV-1 mutants containing additional genes capable of either inhibiting the replication of wild type virus or of killing an HIV-infected cell; and, third, methods of treating a patient infected with HIV will be described.

I. <u>Generation and Characterization of Replication-Incompetent Mutants of HIV-1</u>

The types of HIV-1 linker substitution mutants which have been generated thus far are listed in Table 1. The experimental details for the generation and characterization of six of these mutants is documented below.

Table 1

MUTATIONS IN THE HIV-LTR

Mutant		Known or Putative Site in Mutated Region
Linker Substitution Mutants:		
-453/-436	NXS	
-435/-416	NXS	
-417/-400	NXS	
-399/-382	NXS	
-381/-364	NXS	"Site A"
-363/-346	NXS	AP-1
-345/-328	NXS	AP-1, "Site B," NRE
-327/-310	NXS	NRE
-309/-292	NXS	NRE
-291/-274	NXS	NRE, NFAT-1
-273/-256	NXS	NRE, NFAT-1, IL-2
-255/-238	NXS	NRE, IL-2
-237/-220	NXS	NRE, IL-2
-219/-202	NXS	NRE
-201/-184	NXS	NRE
-183/-166	NXS	NRE,IL-2Rα, Neg. Regulatory Factor, USF-1, USR
-165/-148	NXS	USR
-147/-130	NXS	TCF-1α
-129/-112	NXS	
-111/-94	NXS	NF-$_K$B, HIVEN86A, EBP-1
-93/-76	NXS	NF-$_K$B, HIVEN86A, EBP-1 (Sp1- 1st 2 bases)
-75/-58	NXS	Sp1
-57/-40	NXS	Sp1
-39/-22	NXS	TATA
-21/-4	NXS	LBP-1, UBP-1
-3/+15	NXS	LBP-1, UBP-1 (Initiator)
NF-$_K$B Specific Mutants:		
-105/-81	XS+	NF-$_K$B (total mutant)
"mutant $_K$B"		NF-$_K$B (GGG to CTC)

Zeichner et al., *J. Virol.* (1991) 65:2436-2444.

Experimental Methods:

Cells. Human PBLs were harvested as described by Collman et al. (J. Exp. Med. (1989) 170:1149-1163). The cells were stimulated with 5 μg per ml of PHA for 72-96 h and subsequently grown in RPMI-1640 medium supplemented with 15% fetal calf serum, non-essential amino acids, antibiotics, and human recombinant IL-2 (at a concentration of 600 U per ml,

Boehringer Mannheim Corporation, Indianapolis, IN). CEMx174 cells, a stable human T-B lymphoblastoid hybrid cell line, 11.8 cells, a stable hybrid of DF and Molt-4 human T-cell lines and RD, a rhabdomyosarcoma-derived CD4-negative human monolayer cell line, were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and L-glutamine at 37°C in humidified 5% CO2 (Kozbor et al., *J. Immunol.* **(1990)** 144:3677-3683; Salter et al., *Immunogen* **(1985)** 21:235-246; Srinivasan et al., *Proc. Natl. Acad. Sci. USA* **(1989)** 86:6388-6392).

Construction of Proviral Plasmids. The construction of linker scanning mutations in the LTR of HIV-1 is described by Zeichner et al. (*J. Virol.* **(1991)** 65:24336-2444). Briefly, each mutant, containing a substitution of 18 bp of wild-type HIV-1 (HXB2) LTR U3/R sequence with an *Nde*I-*Xho*I-*Sal*I (NXS) polylinker (5′-CATATGCTCGAGGTCGAC-3′)[SEQ.ID.NO:1], was generated using polymerase chain reaction (PCR) and verified by sequencing. Mutants are named using "*n/m* NXS" nomenclature, "n" referring to the LTR nucleotide substituted by the 5′-terminal nucleotide of the NXS polylinker and "m" to the substituted 3′-terminal nucleotide position relative to the transcription start site. Mutated LTR regions examined in this study include: -75/-58 NXS, -93/-76 NXS, -148/-130 NXS, -165/-148 NXS, -183/-166 NXS, and -201/-184 NXS (Figures 1 and 2).

Each NXS LS LTR was cloned into a plasmid containing a single LTR and complete, terminally repetitious proviral coding sequences as described below and as illustrated in Figure 3. The single LTR-proviral fragment was obtained by *BamH*I digestion of pILIC which contains the complete viral coding sequences originally derived from the infectious molecular clone of HIV-1, pNL4-3 (Leonard et al., *J. Virol.* **(1989)** 63:4919-4924) shown in Figure 3A. To avoid the possible complicating effects of mutations in the 3′-LTR on *nef* function, stop codons were inserted in *nef* as follows. The *BamH*I proviral fragment was ligated into the *BamH*I site of the pG3H vector (pGEM3Zf(-), obtained from Promega Corporation, Madison, WI, which was depleted of its *Xba*I to *Hind*III

polylinker sequences). The product was digested with *Xho*I and the overhanging ends were filled in with Klenow fragment, followed by blunt-end ligation with *Xba*I linkers which contain stop codons. Subsequent digestion of the linkers with *Xba*I and religation to recircularize the plasmid introduced stop codons in each reading frame of the *nef*-coding region at the former wild-type *Xho*I site (Figure 3B).

The resulting plasmid, pG3-ILICX, was also employed in subcloning each single-LTR proviral NXS mutant. The *Xba*I-*Sph*I LTR-containing fragment of pG3-ILICX was ligated into a pGEM3Zf(-) vector depleted of its *Hind*III site (Figure 3C). The insert was digested with *Xba*I and *Hind*III, and the resulting wild-type LTR fragment was replaced by each *Xba*I-*Hind*III NXS mutant LTR fragment as well as the wild type LTR sequences of the parent strain used for construction of the NXS mutants (strain HXB2) (Figure 3D). Each resulting mutant pG3H(*n/m*)NXS clone was digested with *Xba*I and *Sph*I to release complete mutant LTR inserts (Figure 3E). These were used to replace the LTR of pG3-ILICX to construct a panel of circularly permuted proviral clones each with single mutant LTRs, called pG3I(*n/m*)NXS (Figure 3F).

Generation and Standardization of Wild Type and Linker-Substitution Mutant HIV-1 Virus Stock. Infectious DNAs were generated from mutant and wild-type HIV-1 as concatemers of the single LTR-containing proviral fragment (Leonard et al., *J. Virol.* **(1989)** 63:4919-4924). First each of the pG3I(*n/m*)NXS and the pG3IWT (wild-type LTR) proviral plasmids were digested with *BamHI*. The 9.08 kilobase proviral fragment was purified by agarose gel electrophoresis and was ligated to regenerate LTRs at appropriate 5′- and 3′-positions flanking restored complete proviral coding sequences (Figure 3G). Concatemerized proviral DNA (10 μg) was ethanol precipitated and transfected into the RD cell line using Lipofectin (25 μg) in Opti-MEM for 6 h at 37°C. The transfection reaction was stopped by adding serum-containing medium (GIBCO/BRL Life Technologies, Inc.) to

the cultures. Within 48 to 72 h, the transfected RD cultures were cocultivated with 1 X 106 CEMx174 cells for an additional 48 to 72 h. Infected CEMx174 cultures were then transferred to T-25 flasks (Costar Corporation, Cambridge, MA) and expanded for 4 to 23 days. Supernatants were harvested, filtered through 0.45 μm pore-size filters, and stored in aliquots at -80°C, which were subjected to a maximum of one freeze/thaw cycle.

Viral stocks were standardized by determining: 1) the concentration of $p24^{gag}$ antigen in the stocks by antigen capture using a commercial antigen capture assay, i.e., enzyme-linked immunosorbent assay (ELISA) kit (Coulter Inc.); and, 2) the level of reverse transcriptase (RT) activity in lysed viral pellets. This was calculated by quantitating trichloroacetic acid-precipitable 3H-dTTP radioactivity. Tissue culture infectious dose-50 ($TCID_{50}$) determinations of the wild-type stocks were performed using the Reid-Munch method (Collman et al., *J. Exp. Med.* **(1989)** 170:1149-1163).

Infection of Cells with Mutant Viruses. Cells were infected in duplicate with viral suspensions containing $5x10^3$ CPM of ^3H-RT activity (approximately 15 ng $p24^{gag}$ of each virus stock, which was equivalent to a multiplicity of infection (MOI) of wild-type HIV-1 of approximately 0.1-0.5 $TCID_{50}$ per cell). Suspensions of 1 X 10^6 cells and virus were incubated at 37°C for 3 h, then briefly trypsinized, washed three times with phosphate-buffered saline, and suspended in 10 ml of complete medium in T-25 flasks. A sample of the cell-free supernatant was obtained as an initial time point. Virus replication was monitored by sampling the supernatant every 3-4 days thereafter and replacing it with fresh medium or expanding the cultures as necessary. Supernatants were stored at -80°C and subsequently assayed for $p24^{gag}$ antigen.

Polymerase Chain Reaction Analysis of Genomic DNA. To ensure that the mutant viruses were capable of entry into cells and of

undergoing reverse transcription despite their low levels of replication, polymerase chain reaction (PCR) analysis was performed. High molecular weight nuclear DNA preparations from infected cultures were analyzed using commercial oligonucleotide primers to detect a 115 bp HIV-1 *gag* sequence. These primers were SK38 (1551/1578): 5′-ATAATCCACCTATCCCAGTAGGAGAAAT-3′ [SEQ.ID.NO:2] and SK39 (1638/1665):5′-TTGGTCCTTGTCTTATGTCCAGAATGC-3′ [SEQ.ID.NO:3] (Perkin-Elmer Corporation, Norwalk, CT). Cells infected with DNase I-treated virus stocks were harvested at 3 and 30 days after infection, and genomic DNA was prepared. Briefly, cell pellets were washed, lysed, and nuclei were digested with proteinase K (0.4 mg per ml) for 4-6 h at 55°C. The lysates were extracted with phenol-chloroform and the DNA was ethanol precipitated. *BamHI*-digested genomic DNAs were amplified in a Perkin-Elmer/Cetus DNA Thermal Cycler using the following protocol: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 70°C for 2 min. The products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining.

To ensure the fidelity of the linker substitution mutations in the progeny viruses, PCR was performed according to the above protocol using primers to amplify a 577 bp LTR fragment containing most of the U3 and R regions (positive-sense 5′ primer (-439/-412): 5′-CTAATTCACTCCCAACGAAGACAAGA-3′ [SEQ.ID.NO:4] and negative-sense 3′primer (+112/+138): 5′-TCTCTAGTTACCAGAGTCACACAACAG-3′ [SEQ.ID.NO:5]. The amplified 577 bp LTR fragment was sequenced directly to confirm that spontaneous mutations did not occur during viral propagation (Perkin-Elmer Corporation, Norwalk, CT).

Experimental Results:

The effects of discrete LS mutations on the HIV-1 LTR on CAT reporter gene transcription in transient transfections of Jurkat cells has been described (Zeichner et al., *J. Virol.*

(1991) 65:2436-2444). Substitution of certain LTR regions with an 18 bp NXS linker had variable effects on transcriptional activity depending on the cellular conditions (Zeichner et al., *J. Virol.* (1991) 65:2436-2444). These mutations affected regions that had not previously been associated with significant positive transcriptional activity (e.g. -201 to -130).

Six NXS mutant LTRs were used in the studies described below. Four LS mutants, which together span nucleotides -201 to -130, altered certain transcription factor binding sites and in LTR-CAT transfection studies displayed between 25% and 70% of wild-type CAT activity, depending on the mutant (Figure 2, see mutants: -147/-130 NXS, -165/-148 NXS, -183/-166 NXS, and -201/-184 NXS). Two other mutants, -75/-58 NXS and -93/-76 NXS, which affect the NF_KB and Sp-1 binding sites, were selected as controls since they dramatically decreased LTR-mediated transcription (Leonard et al., *J. Virol.* (1989) 63:4919; Parrott et al., *J. Virol.* (1991) 65:1414-1419; Ross et al., *J. Virol.* (1991) 65:4350-4358).

Generation of Wild-Type and Linker-Substitution Mutant LTR HIV-1 Stocks. Cell cocultivation techniques, such as those described here, have been found to be effective in producing stocks of viruses with debilitating LTR mutations. The data presented below demonstrates that a cocultivation procedure using RD and CEMx174 cells results in the production of stocks of the NXS LTR mutant viruses which cannot otherwise be propagated in single cell lines. Thus, concatemerization of single LTR-containing proviral DNA (Figure 3G) followed by transfection into RD cells and subsequent co-cultivation with CEMx174 cells proved to be an efficient method for generating mutant virus stocks. Although mutants -93/-76 NXS and -75/58 NXS failed to replicate in subsequent individual cell type infections with cell-free inocula, all six mutant viruses were able to replicate to levels resulting in detectable cytopathic effects (CPE), primarily syncytia formation, under co-cultivation conditions. To avoid degradation of viral

infectivity due to prolonged co-cultivation, the more rapidly replicating viruses were harvested earlier than those with lower levels of CPE and were frozen at -80°C. After harvesting, virus stock titers were standardized by measuring the level of $p24^{gag}$. These data were supplemented with the results of analysis of levels of RT activity of virus pellets. Each virus stock contained significant amounts of both $p24^{gag}$ and RT and had consistent RT:$p24^{gag}$ ratios. Due to the differences in the titers of some mutant viruses, we were able to attain a maximum equivalent inoculum in subsequent individual cell type infections of approximately 15 ng per ml $p24^{gag}$. This inoculum resulted in an infectivity of the wild-type HIV-1 stocks in CEMx174 cells of 105-106 $TCID_{50}$ per ml, corresponding to MOIs of 0.1-0.5 $TCID_{50}$ per cell.

Replication of Mutant Viruses in Single Cell Cultures. Equivalent inocula of wild-type and mutant virus stocks were used to infect individual cell types. The production of viral antigen in the supernatant was quantified using a $p24^{gag}$ antigen capture assay with a sensitivity of 10-20 pg per ml. The results were reproducible among separate infections; typical results are shown in Figure 4A, B, and C. PBLs and 11.8 cells supported the replication of only wild-type and mutant -183/-166 NXS viruses. Wild-type HIV-1 achieved peak $p24^{gag}$ levels of approximately 10^2 ng per ml 10 days post-infection (p.i.) in PBLs (Figure 4A) and 10^3 ng per ml 48 days p.i. in 11.8 cells (Figure 4B). Mutant -183/-166 NXS consistently demonstrated slower kinetics than wild-type and occasionally produced lower concentrations of viral antigen, although the differences were barely statistically significant. In PBL infections, -183/-166 NXS yielded 10 ng per ml $p24^{gag}$ 10 days p.i., and 102 ng per ml 50 days p.i. in 11.8 cells (Figure 4B). None of the other mutant viruses produced significant levels of $p24^{gag}$ in either of these differentiated T-cell types.

Not surprisingly, the cell line used to raise viral stocks in co-cultures, CEMx174, demonstrated the greatest permissiveness for virus replication in single cell type

infections, achieving $p24^{gag}$ levels of 10^3 ng per ml by 12 days p.i. for both wild-type and mutant -183/-166 NXS (Figure 4C). CEMx174 was the only cell line in which mutant viruses -147/-130 NXS, -165/-148 NXS, and -201/-184 NXS replicated sufficiently to detect antigen in the supernatant (see below). Mutants -93/-76 NXS and -75/-58 NXS failed to replicate in any of the individual cell type infections.

Infection of Individual Cell Types Reveal Different Replication Phenotypes of Viral Mutants. Based on their growth characteristics in PBLs, 11.8, and CEMx174 cells, the six mutant viruses were classified into three broad phenotypes: 1) wild-type replication in all three cell types, 2) severely reduced replication in all three cell types, and 3) severely reduced replication in PBLs and 11.8 cells, and delayed replication kinetics in CEMx174 cells.

1) Wild-type replication phenotype in all cell types. Mutant -183/-166 NXS consistently demonstrated a replication phenotype similar to wild-type in all three cell types. The mutant linker substitutes 8 bp of the possible upstream repression sequence (URS, -188 to -176) and 8 bp of the binding site of USF-1 (-173 to -160), a putative negative regulatory protein (Figure 2) (Garcia et al., *EMBO J.* **(1987)** 6:3761-3770; Lu et al., *J. Virol.* **(1990)** 64:5226-5229). Nonetheless, the mutant -183/-166 NXS expressed near-wild-type levels of $p24^{gag}$ in a comparable time course. Hence, mutations within nucleotides -183 to -166 alone do not produce a phenotype indicative of negative regulatory function for this region.

2) Severely reduced replication phenotype in all cell types. LS mutation of NFkB- and Sp1-binding sites caused drastic diminution of viral replication in all three cell types (Figure 4). Mutant -93/-76 NXS substitutes the entire 3′-proximal NFkB-binding site (NFkB-1, -91 to -81) and 3 bp of the 5′-proximal Sp1-binding site (Sp1-III, -78 to -67) (Figure 2). Mutant -75/-58 NXS replaces most of both the 5′-proximal

(Sp1-III, -78 to -67) and middle Sp1-binding sites (Sp1-II, -66 to -57) (Figure 2). Their dramatic effects in viral infections agree with the transfection analysis, in which these mutations had the greatest effects on LTR-CAT expression under all conditions tested (as low as 4% and 21% of wild-type, respectively; Figure 2) (Zeichner et al., *J. Virol.* (1991) 65:2436-2444). The data presented here illustrate the absolute requirement of these *cis*-acting elements for efficient viral replication in these cell types.

3) Severely reduced replication in PBLs and 11.8 cells, but delayed replication kinetics in CEMx174. Although PBLs and 11.8 cells failed to support infections with viruses -201/-184 NXS, -165/-148 NXS, or -147/-130 NXS, these viruses replicated successfully in the highly permissive CEMx174 cells, both in the initial transfection/co-cultivation and in the subsequent individual cell type infections at lower multiplicities (Figure 4C). Since significant replication of these mutants was not observed in either PBLs or 11.8 cells, the substituted regions appear necessary to initiate viral growth in these more differentiated T-cell types. Hence, mutations such as these, in addition to similar mutations in the region of nucleotides -201 to -130, are the preferred mutations for the generation of the CRI viruses of the invention.

Mutant Viruses do not Revert to Wild-Type. To exclude the possibility of reversion to a wild-type phenotype as an explanation for the growth of -183/-166 NXS, and for the delayed kinetics observed in CEMx174 infections with mutants -165/-148 NXS, -147/-130 NXS, and -201/-184 NXS, reinfection of CEMx174 was performed with mutant virus stocks obtained late during productive infection of CEMx174 cells (i.e. up to 60 days p.i.). The kinetics of virus growth in reinfected cells was indistinguishable from that in cells infected with the parental mutant stocks, supporting the fact that these viruses retained their mutant phenotype.

In order to ensure that each mutant virus was capable of entry into cells and integration into host cell DNA, PCR was performed on DNA samples obtained from cells infected with DNase I-treated virus stocks. High molecular weight nuclear DNA was extracted from 10^6 PBLs at 3 and 30 days p.i. and an HIV-1-specific 115 bp target *Gag* sequence was amplified. The presence of HIV-1 sequences in each sample, as detected by ethidium bromide staining in agarose gels, confirmed that the observed phenotypes of mutant viruses were not due to an inability to infect the target cells nor to defects in viral entry. These results are consistent with integration of the proviral DNA into host cell DNA sequences.

To check for the original NXS mutations and for other possible alterations of the LTR sequence, infected cell DNA preparations were analyzed by PCR using a primer pair that amplified 577 bp of the LTR sequence (-439 to +138). Each mutant LTR fragment was sequenced. The sequence data confirmed not only the presence of the NXS linkers but also the absence of any additional potentially compensatory LTR mutations, ensuring that the observed replication phenotypes were attributable to the original NXS mutations.

To summarize the genetic characteristics of the mutant viruses including the mutations introduced into the original starting material as described in Zeichner et al., (*J. Virol.* **(1991)** 65:2436-2444), stop codons were placed upstream of the linker substitution inserts into the *nef* reading frame and an *Xba*I site encoding these stop codons was inserted to faciliatate cloning of the NXS mutations. The use of the stop codons eliminates the possibility that unknown and/or variable fusion proteins can be expressed from this region. The *nef* coding region is further disturbed by the NXS mutations as well as by a small (61 bp) segment of cellular sequence upstream of the LTR which derives from the proviral source of the LTR used to make the original set of NXS mutations.

It is known that the *nef* gene product affects the pathogenesis of Simian Immunodeficiency Virus (SIV). Monkeys infected with a *nef* minus virus remain healthy and do not

develop simian AIDS (Daniel et al., *Science* (1992) 258:1938-1941; Kestler, *Cell* (1991) 65:651-662). It is assumed, although it cannot be tested directly, that the *nef* gene product similarly affects the pathogenesis of HIV in humans. Hence, the manner in which the mutants of the invention have been constructed has provided additional safety features in terms of their potential pathogenesis and in terms of any therapeutic effect the *nef* minus mutation might impart. Furthermore, because the viruses encode multiple mutations, it is highly unlikely that they are capable of reverting back to a wild-type phenotype and indeed the data presented above indicates that they do not revert in tissue culture.

Yet other mutations may also be introduced into the genome of the mutants of the invention to provide additional safeguards against reversion. For example, the Tat gene encoded by the CRI viruses is dispensable and can be mutated such that it is non-functional. This can be accomplished by first introducing a mutation into the Tat gene on a plasmid template and then incorporating that mutation into the proviral DNA of a CRI virus using ordinary recombinant DNA and virological technologies familiar to those in the art. Such viruses can be propagated on Tat-expressing cell lines the generation of which is described below.

The results presented above clearly document that analysis of the *in vivo* effects of LS mutations in the LTR is essential for determining which regions of the LTR, when mutated, have an effect on virus replication. Transfection analysis alone is not sufficient to predict which mutations might have the greatest effect on virus replication. For example, mutations encoded by -75/-58 NXS, -93/-76 NXS, -147/-130 NXS, and -165/-148 NXS had a much more marked effect on virus replication in cells than could have been predicted from the results of the transfection studies (Zeichner et al., *J. Virol.* (1991) 65:2436-2444).

Therefore, the efficacy of viral mutants in *in vivo* replication assays cannot be accurately predicted from transfection studies. Further, the data demonstrate that even

the most debilitating mutations can be introduced into viruses which can then be propagated by appropriate cocultivation methods.

The invention should not be construed as being limited to the CRI HIV mutants described above. Rather, the invention encompasses any mutant of HIV which comprises a mutation in the LTR which renders the virus conditionally replication-incompetent in the absence of wild type Tat protein. Such mutations include the insertion of linkers other than the NXS linker utilized in the studies described above; the substitution of specific nucleotides with sequences which do not form restriction enzyme cleavage sites as is the case with linkers; the generation of specific point mutations; and, other deletion and insertion mutations.

The invention should also not be construed as being limited solely to HIV-1. Because of the similarities in the sequence of HIV-specific LTRs, any strain of HIV, including HIV-2, can be mutated using the techniques described above, such that CRI viruses are generated with properties similar to those described above. Furthermore, additional mutations can then be constructed in the genome of these viruses using the techniques described below, such that CRI viruses derived from any strain of HIV which are useful for the treatment of HIV-infected individuals are generated.

Tat-Induced Activation of Conditional Replication-Incompetent Viruses

The mutant viruses of the invention which are severely debilitated in their replication-competency in PBLs and 11.8 cells (-201/-184NXS, -165/-148NXS, and -147/-130NXS) are mutated in a region of the LTR which is not considered to be necessary for activation by the Tat protein. While nucleotides -1 to -125 have been shown to be required for Tat activation, nucleotides outside of this region are dispensable for this function (Zeichner et al., *J. Virol.* **(1991)** 65:2436-2444). In fact, the mutated LTR sequences of the invention are capable of being activated by Tat in transient transfection assays

(Zeichner et al., *J. Virol.* **(1991)** 65:2436-2444). In order to establish that these sequences also respond to Tat in the context of the viral genome, each mutant virus constructed as described above, can be tested in cells which stably express wild type Tat. The data can be compared with those obtained in mutant infected cells which do not express Tat. The degree to which the mutants replicate is assessed by measuring the levels of $p24^{gag}$ antigen and RT activity in the cultures using the methods described above.

Tat-expressing cells useful for these studies are available (Rosen et al., *Cell* **(1985)** 41:813-823), or can be generated using ordinary molecular biology protocols familiar to those in the art and described for example, in the Molecular Cloning Manual (Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor, New York), or they can be generated in a manner similar to that described for the stable expression of the transdominant Rev protein (Malim et al., *J. Exp. Med.* **(1992)** 176:1197-1201).

In the latter example, essentially, the Tat gene is cloned into a plasmid encoding a recombinant retrovirus capable of expressing an antibiotic resistance marker, i.e., neomycin resistance. This plasmid is then transfected into a virus-packaging cell line such as, but not limited to, the ecotropic Ψ CRE virus packaging cell line, and cells so transfected are selected for their resistance to the appropriate antibiotic, e.g., G418 (Danos et al., *Proc. Natl. Acad. Sci. USA* **(1988)** 85:6460). Following incubation for approximately two days, filtered supernatant is collected from the cultures and used to infect an amphotropic cell line such as the Ψ CRIP-packaging line, which is then selected in G418 at a concentration of approximately 1 mg/ml. Clones of cells are selected for their resistance to G418 and for their ability to produce virus. Viral supernatants obtained from these cultures are incubated with the specific cell line in which stable expression of Tat is required. Following incubation, clones of cells are selected for G418 resistance and are tested for the

presence and expression of the Tat gene using molecular or immunological techniques to detect Tat-specific DNA, RNA or protein familiar to any ordinary molecular biologist and described for example in the Molecular Cloning Manual (Sambrook et al., Supra).

Generation of Conditional Replication-Incompetent Viruses Encoding Additional Genes

Conditional replication-incompetent HIV-1 mutants encoding additional genes can be constructed in a manner identical to that described above for the generation of the original conditional replication-incompetent mutants. A plasmid encoding a mutation in the LTR sequence can be further manipulated using ordinary molecular biological technology familiar to those in the art such that a heterologous gene or an alternate viral gene is positioned in the mutated HIV-1 genome so as to be expressed only when the mutated LTR is activated by Tat. Essentially, a plasmid encoding a terminally repetitious provirus containing a single mutated LTR (Figure 3) is linearized at the site into which the gene will be inserted and a fragment encoding that gene is then ligated into this site. The resulting plasmid is separated from unligated material and an infectious provirus is generated from this plasmid as described above. The precise location with respect to the terminally repetitious single LTR provirus into which such a gene might be placed is determined by analysis of the known sequence of the virus, the sequence of that particular gene and the position of the alternate viral gene if this is the gene of choice.

The specific site into which the gene will be inserted will depend on the particular gene to be inserted. If a viral gene is replaced with an alternate form of that gene, e.g., a mutated viral gene encoding a transdominant viral protein, then the mutated gene would be inserted in place of that viral gene, at a position in the genome in which the wild type form of the gene normally resides. For example, a Rev-specific sequence encoding a transdominant form of that protein can be inserted

into the viral genome by simply replacing the *BamHI* to *XhoI* fragment in the genome of the replication-incompetent virus with the comparable *BamHI* to *XhoI* fragment encoding the transdominant form of Rev (Malim et al., *Cell* **(1989)** 58:205-214). In the CRI viruses, the *XhoI* site has been converted to an *XbaI* site as described above. Hence, the *XhoI* site in the *BamHI* to *XhoI* fragment encoding the transdominant Rev is replaced with an *XbaI* linker for proper insertion and maintenance of the stop codons in *nef*. Other transdominant alternative viral genes can be inserted into background of the replication incompetent viral genome in a similar manner.

If a heterologous non-viral gene is to be inserted, either under the control of the viral LTR or another internal promoter, this gene can be positioned in the viral genome such that it replaces viral sequences which are dispensable for growth of the virus in cell culture. An example of sequences which are dispensable for viral growth in culture are the *nef* coding sequences (Daniel et al., *Science* **(1992)** 258:1938-1941). Since *nef* is expressed to very high levels during replication of HIV, it is expected that an additional gene inserted into the *nef* coding region would also be expressed to high levels.

When an alternate viral gene is inserted into a replication incompetent viral genome, the resulting recombinant virus may be incapable of replication using the cocultivation techniques described above. In this case, supporting cell lines can be constructed which constitutively express a recombinant gene capable of complementing the defect in the mutated virus thus facilitating its replication. For example, when the wild type Rev gene is replaced with a transdominant mutant form of the gene, the resulting virus can only be propagated on cell lines which constitutively express the wild type form of a similar viral protein, e.g., the wild form of the Rex gene product encoded by the human T cell leukemia virus type 1 (HTLV-1) (Bachmayer et al., European Patent Application No. 90109892.1). Construction of such supporting cell lines is accomplished in a manner similar to that described above, or by

following other published protocols (e.g., Rosen et al., *J. Virol.* (1986) 57:379-384; Chen et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:7678-7682).

Genes which are suitable candidates for mediating inhibition of wild-type HIV-1 replication include those encoding 1) proteins which suppress viral transcription, gene expression or replication; 2) antisense RNA specific for a particular essential viral mRNA or a structural feature of the viral RNA, which when disrupted, would limit virus replication; 3) catalytic RNA (ribozyme) specific for an essential viral mRNA or a structural feature of viral RNA which when disrupted would limit viral replication; and 4) a toxin, which when expressed would kill an infected cell.

Preferably, these genes encode viral proteins which are transdominant with respect to the wild-type protein. For example, genes encoding a transdominant Rev protein as described above, an HIV-specific Tat protein (Pearson et al., *Proc. Natl. Acad. Sci. USA* (1990) 87:5079-5083) or a transdominant HIV-specific Gag protein (Trono et al., *Cell* (1989) 59:113) are suitable genes which when expressed may competitively inhibit the normal function of the wild-type protein and thereby inhibit virus replication. Alternatively, transdominant forms of the HTLV-1 Tax and Rex genes may also be used (Bachmayer et al., European Patent Application No. 90109892.1). Potentially, any transdominant gene encoded by HIV, or a transdominant encoded by a heterologous virus, which gene functions to inhibit HIV replication, can be used.

A gene which is most useful in the invention is a gene encoding a transdominant HIV-1 Rev protein (Malim et al., *Cell* (1989) 58:205-214). It is known in the art that functional expression of Rev is required for replication of HIV-1. However, it is also known that particular mutations in the carboxy-terminal portion of Rev not only render Rev defective, but also generate Rev proteins capable of competitively inhibiting wild-type Rev function (Malim et al., *Cell* (1989) 58:205-214). Thus, CRI viruses constructed such that they encode a Tat-activatable LTR, which when activated express a

transdominant Rev protein, have the potential to inhibit replication of a Tat-producing wild type virus present in the same cell, because the transdominant Rev protein will competitively inhibit wild type Rev and effectively abolish this essential function for virus replication (Malim et al., *J. Exp. Med.* **(1992)** 176:1197-1201)

CRI viruses can also be constructed which encode antisense RNA specific for a particular viral mRNA or a structural feature of viral RNA which when disrupted, would limit replication of the virus. An example of such a region is an antisense RNA complementary to the HIV-1 TAR sequences. These sequences are essential for viral transcription and efficient polyadenylation of viral RNA. Cultured cells which express antisense TAR specific sequences are diminished (by as much as 90%) in their ability to support wild type HIV-1 replication (Chatterjee et al., *Science* **(1992)** 258:1485-1488). Insertion of an antisense TAR sequence into a CRI HIV requires that the resident TAR region, a stem loop structure in the RNA, be appropriately modified in order that it is not affected by the antisense RNA. Such modifications of the viral sequences which retain transcriptional and polyadenylation activity have been characterized (Feng et al., *Nature* **(1988)** 334:165-167; Gilmartin et al., *EMBO J.* **(1992)** 11:4419-4428).

CRI viruses, constructed such that they encode a ribozyme specific for a viral mRNA or an essential structural feature of viral RNA, may also be generated. A ribozyme can be designed which is capable of recognizing and cleaving specific viral RNA sequences using a mechanism similar to that utilized by RNase P in the processing of precursor tRNA in *E. Coli* (Guerrier-Takada et al., *Cell* **(1983)** 35:849; Pace et al., *J. Biol. Chem.* **(1990)** 265:3587; Altman, *J. Biol. Chem.* **(1990)** 265:20053; Haas et al., *Science* **(1991)** 254:853-856). Such a cleavage event would render the viral target sequence fragmented and therefore functionless. As indicated above, insertion of a ribozyme-encoding sequence into a CRI HIV requires that the genome of the CRI virus be appropriately modified in order that the ribozyme cannot affect that genome.

CRI viruses can also be constructed encoding proteins capable of killing an infected cell which in turn results in inhibition of wild type virus replication. Genes encoding such proteins are introduced into CRI viruses in a manner similar to that described above for genes that inhibit wild-type HIV replication. Suitable genes include genes encoding toxins or other inhibitors of cellular function. Preferably, the products of such genes should be capable of affecting only those cells in which they reside. An example of such a gene is that which encodes the Ricin A subunit. The entire Ricin toxin comprises two subunits, A and B. Ricin A induces its toxic effect on cells by inhibiting cell-specific translation in the absence of the Ricin B subunit. Since the function of the B subunit is to facilitate attachment and entry of the A subunit into cells, the A subunit can only affect the cells in which it resides in the absence of the B subunit. Cell free A subunit is therefore harmless to cells in the absence of the B subunit (Wawrzynczak, British J., *Cancer* **(1991)** 64:624-630).

Additional Viruses Useful in the Invention

It is well known in the art that HIV-1 infects many cell types in addition to lymphocytes. In fact, infection of a variety of cell types may contribute to the diverse manifestations of the disease (Fauci et al., *Science* **(1988)** 239:617-622). The data presented above demonstrate that CRI viruses encoding defined mutations in specific regions of the HIV-1 LTR are replication-incompetent in the absence of Tat in cells of lymphoid origin. It is possible to construct similar replication-incompetent Tat-dependent viruses capable of infecting other cell types, for example, myeloid cells or glial cells.

To accomplish this, a wild type HIV-1 genome obtained from a macrophage tropic virus is used as the starting material. Mutations are introduced into the LTR region (using the methods described above) which confer the property of replication-incompetence to the virus. Alternatively, a minimal determinant for macrophage tropism has been mapped to

the third variable region of the gp120 glycoprotein (O'Brien et al., *Nature* **(1990)** 348:69-73). This can be inserted into the proviral DNA of a CRI virus following removal of the corresponding sequence from the CRI virus. Using either method, a CRI virus can be generated which is both T cell and macrophage tropic thus providing broader therapeutic benefit to the patient.

Additional genes can then be inserted into these viruses which are capable of either inhibiting wild-type virus replication or of killing an infected cell using the above described methods. As new strains of virus emerge with altered cell-tropic properties, it will be possible using the methods and compositions of the invention, to construct additional viral mutants capable of infecting a variety of cell types, which viruses have therapeutic potential. Since the sequence of the viral LTR influences tissue tropism (Corboy et al., *Science* **(1992)** 258:1804-1808), as more data become available, it will be possible to design replication-incompetent viral mutants with altered LTR sequences capable of acting as therapeutic agents in specific tissues.

Methods of Improving the Safety of Mutant Viruses Useful as Therapeutic Agents

As stated above, the replication-incompetent mutants of the invention encode multiple mutations which serve to ensure that these viruses are incapable of reversion to a wild-type phenotype. These include the LTR mutation conferring replication incompetence in the absence of Tat, the *nef* mutation and the alternate viral gene or heterologous gene capable of inhibiting virus replication when expressed. In order to ensure that these viruses are unable to replicate in a Tat-independent manner in other cell types, additional mutations may be introduced into different regions of the LTR which have cell-specific or differentiation-dependent effects on transcriptional activity. These regions map not only to the 3'-portion of the LTR where many transcriptional control elements have been identified, but also to regions of the LTR

which were previously thought to have little or no transcriptional function (Zeichner et al., *J. Virol.* (1992) 66:2268-2273). It is these latter regions which are most useful in the instant invention.

For example, mutations introduced into specific sites in the 5-prime region of the LTR have been shown to be required for transcription in differentiated embryonyl carcinoma cells exhibiting a neuronal phenotype in transient transfection assays (Zeichner et al., *J. Virol.* (1992) 66:2268-2273). Thus, CRI mutant viruses can be constructed which encode all of the features documented above, and in addition, which are mutated so as to be potentially replication-incompetent in neuronal cells. The introduction of this additional mutation should not affect the replication capabilities of the mutants in lymphoid or myeloid cells since the regions of the LTR in which the various mutations would reside are separated from one another. As more information becomes available on which specific regions of the LTR are involved in transcriptional control in the diverse cell types shown to be infected with HIV, it will be possible to target these regions and introduce additional mutations which further ensure the safety of the mutants as therapeutic agents and as vaccines. An added advantage of these additional mutations is the fact that they have the potential to broaden the number of cell types in which the mutant viruses can function as therapeutic agents.

In order to generate viruses encoding multiple mutations, each of the desired mutations is introduced into the HIV-1 genome on a plasmid template using the methods described above and any other ordinary recombinant methodology familiar to those in the art and described , for example, in the Molecular Cloning Manual (Sambrook et al., Supra). Then, using the transfection and cocultivation methods described above, viruses containing the desired mutations can be generated, screened and characterized.

Methods of Treating Patients Infected with HIV-1

As noted above, the mutant viruses of the invention are useful as therapeutic agents for the treatment of HIV-infected individuals because they have the capacity to infect the same cells in the patient which are infected with wild-type virus; and, when activated by Tat expressed from the wild-type viral genome, the mutants are capable of inhibiting replication of the wild-type virus, or of killing the infected cell. In either case, the production of new progeny viruses will be limited or completely inhibited. Consequently, the extent of infection of individuals with HIV will be retarded or may in fact be eliminated. Such therapies are vital for HIV-infected patients because treatment during the asymptomatic period delays the decline in the number of CD4 positive cells characteristic of the onset of AIDS, and therefore may prolong life. Furthermore, the therapeutic agents of the invention may also be useful in prolonging life even after the patient has progressed to AIDS in that the rate at which their immune function declines may be retarded following treatment.

The therapeutic agents of the invention are administered to HIV-infected patients in one of several ways. In general, the mutants are delivered to the hematopoietic cells of the patient in either the blood or the bone marrow.

1) Direct Inoculation

Blood cells and other susceptible cells of an HIV-positive patient are infected with the mutants of the invention by simple direct inoculation of virus into the patient's blood stream. This can be accomplished by injection of CRI viruses suspended in a pharmaceutically acceptable carrier using a syringe, or by injection using an intravenous apparatus. A significant advantage to this approach is that pantropic viruses, capable of infecting many different cell types can be used because, once free in the blood stream, they can infect any number of the appropriate cells. For example, if a patient is inoculated with a CRI virus which is both macrophage and T cell tropic (Gartner et al., *Science* **(1986)** 233:215-219; Cameran et al., *Science* **(1992)** 257:383-387), this

virus is capable of infecting macrophages, T cells and, in addition, glial cells through the utilization of the galactosyl ceramide receptor (Harouse et al., *Science* (1991) 253:320-323). This approach may require the patient to undergo frequent injections with large quantities of virus. It is anticipated that the patient may receive a dose of virus in the range of 10^3 to 10^8 $TCID_{50}$, either daily or at periodic intervals, the frequency of which will depend upon the extent of the HIV-1 infection in the individual, their immune status and whether the exhibit symptoms of AIDS and other factors. The exact therapeutic protocol will therefore be empirically designed for each individual patient and will be apparent to those skilled in the art of treatment of HIV-infected patients.

The cells of the patient may also be infected with a mutant virus *in vitro* and then subsequently be returned to the patient. Human gene therapies which are known in the art have proved to be encouragingly successful for the treatment of diseases such as Severe Combined Immunodeficiency (SCID), (Anderson, *Science* (1992) 256:808-813).

2) Lymphocyte Therapy

The mutants of the invention may be administered to an HIV-infected patient following the general protocol useful for the treatment of children with adenosine deaminase deficient SCID (Culver et al., *Transplantation Proceedings* (1991) 23:170-171). Essentially, blood cells are obtained from the patient from which a subpopulation of T lymphocytes are isolated. The numerous techniques for separating T lymphocytes from other cells of the blood are well known in the art and are described, for example, in Culver et al., Transplantation Proceedings 23:170-171; Culver et al., *Human Gene Therapy* (1991) 2:107-109. The T cells are infected *in vitro* with one of the mutant viruses of the invention using the methods described above for infection of cells in culture. Following a period of incubation, the infected cells, suspended in a physiologically acceptable carrier, are then returned to the patient's blood stream.

The amount of virus required for efficient infection of T lymphocytes *in vitro* is in the range of 0.1 to 50 $TCID_{50}$. The number of T cells required for efficient treatment of the patient may range from 10^1 to 10^8 cells per kg body weight. Patients will require repeated treatment when lymphocyte therapy is the preferred method. The frequency of treatment will depend upon the extent of the HIV-1 infection in the individual, their immune status, whether they exhibit symptoms of AIDS and other factors. For this reason, the exact therapeutic protocol will be empirically designed for each individual patient and will be apparent to those skilled in the art of gene therapy and treatment of HIV-infected patients.

3) Monocyte and/or Lymphocyte Therapy

In vitro infection of monocytes obtained from an HIV-1 infected patient may also serve as an efficient route of administration of the mutants of the invention to the patient. Freshly isolated human blood monocytes can be maintained *in vitro* for several hours to days before they differentiate into a macrophage phenotype (Albers et al., *Virology* (1989) 169:466). Thus, monocytes obtained from a sample of blood from the patient using ordinary immunological techniques may be infected with the appropriate virus in a manner similar to that described above. Cells so infected are then returned to the patient. If a the viral mutant of choice is capable of infecting both T cells and monocytes, then both cell types may be infected at the same time providing a increased dose of virus with minimal discomfort to the patient. The amount of virus to be used and the frequency of administration is similar to that described above.

4) Bone Marrow/ Progenitor Cell Therapy

It is known in the art that HIV-1 infected individuals often harbor virus in bone marrow progenitor cells. It is also known that bone marrow cells when isolated *in vitro*, can be infected with HIV (Folks et al., *Science* (1988) 242:919-922; Scadden et al., *Blood* (1990) 76:3317-3322; Folks, *Blood* (1991) 77:1625-1626; Kitano et al., *Blood* (1991) 77:1699-1705). Infection of such cells with the mutants of the invention

therefore provides an additional method of therapy for HIV-infected individuals. Cells obtained from the individual can be isolated and infected with the mutants of the invention *in vitro* using the general methods described in Folks et al. (*Science* **(1988)** 242:919-922), Scadden et al., (*Blood* **(1990)** 76:3317-3322), Folks, *Blood* **(1991)** 77:1625-1626) and Kitano et al. (*Blood* **(1991)** 77:1699-1705). Infected cells are then returned to the bone marrow of the individual where they will continue through the differentiation process. Peripheral blood cells which arise following differentiation of mutant-infected progenitor cells will provide a constant and renewable source of mutant virus-protected lymphocytes and monocytes. An advantage of this approach is that the patient should not require repeated inoculation of viruses. In addition, if a mutant infected cell harbors a wild type form of the virus which is activated to replicate and therefore expresses Tat, or, if such a mutant infected cell is subsequently infected with a wild type, replicating, Tat expressing HIV, then the LTR of the mutant is activated and in turn drives expression of a gene in the mutant capable of either inhibiting replication of wild type HIV or of killing the infected cell. Overall, this type of therapy has an advantage over the lymphocyte or monocyte therapies described above in that it has greater potential as a long term therapy, wherein the frequency treatment with the mutant viruses might be reduced.

Bone marrow cells can be obtained from HIV-infected individuals using established protocols available to those in the art and described for example in Kitano et al. (*Blood* **(1991)** 77:1699-1705), or Folks et al. (*Science* **(1988)** 242:919-922). Essentially, a suspension of cells is obtained from the bone marrow of a patient using conventional techniques. If necessary, the population of cells of interest may be separated from the remainder of the cells in the sample using a combination of techniques including centrifugation and flow cytometry. Cells so isolated are then infected with the mutants of the invention as described above and are returned to the bone marrow of the patient following infection.

The amount of mutant virus required for infection of bone marrow cells from an HIV-infected individual and the number of cells to be used during each therapeutic treatment can be determined empirically by one skilled in the art of gene therapy. Because the extent of treatment of any HIV-infected individual will vary depending upon the virus load they carry, their level of immune competence, their age, the number of years they have been infected and other factors, the exact protocol to be used in any one individual will be determined on a case by case basis. In general, the dosage of virus and the number of cells to be used will be within the range given above for the lymphocyte therapy approach.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Alwine, James C.
Gonzales-Scarano, Francisco
Zeichner, Steven L.
Malim, Michael H.

(ii) TITLE OF INVENTION: MUTANTS OF HIV FOR SUPPRESSION OF HIV INFECTION

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: One Liberty Place - 46th Floor
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19103

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/002,609
(B) FILING DATE: 11-JAN-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Leary, Kathryn
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(C) REFERENCE/DOCKET NUMBER: UPN-1610

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (215) 568-3439
(C) TELEX: N/A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CATATGCTCG AGGTCGAC

(2) INFORMATION FOR SEQ ID NO:2:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(i) SEQUENCE CHARACTERISTICS:
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What is Claimed:

1. A Human Immunodeficiency Virus, wherein the genome of said virus comprises a long terminal repeat sequence comprising a mutation which renders said virus conditionally replication-incompetent in the absence of wild-type Tat protein supplied in trans.

2. The Human Immunodeficiency Virus of claim 1, wherein said mutation is a linker substitution mutation.

3. The Human Immunodeficiency Virus of claim 2, wherein said linker substitution mutation is positioned between nucleotides -453 to -57.

4. The Human Immunodeficiency Virus of claim 3, wherein said linker substitution mutation is positioned between nucleotides -453 to -111.

5. The Human Immunodeficiency Virus of claim 4, wherein said linker substitution mutation is positioned between nucleotides -435 to -111.

6. The Human Immunodeficiency Virus of claim 5, wherein said linker substitution mutation is positioned between nucleotides -201 to -130.

7. The Human Immunodeficiency Virus of claim 6, wherein said virus is selected from the group consisting of -147/-130 NXS, -165/-148 NXS or -201/-184 NXS.

8. The Human Immunodeficiency Virus of claim 1, wherein said virus is conditionally replication-incompetent in peripheral blood lymphocytes.

9. The Human Immunodeficiency Virus of claim 1 wherein said virus is conditionally replication-incompetent in 11.8 cells.

10. The Human Immunodeficiency Virus of claim 1, wherein said virus further encodes a gene capable of inhibiting replication of wild-type Human Immunodeficiency Virus.

11. The Human Immunodeficiency Virus of claim 10, wherein said gene is a transdominant gene.

12. The Human Immunodeficiency Virus of claim 11, wherein said gene is Rev.

13. The Human Immunodeficiency Virus of claim 11, wherein said gene is selected from the group consisting of Rev, Gag, Tat, Tax or Rex.

14. The Human Immunodeficiency Virus of claim 10, wherein said gene encodes a product selected from the group consisting of antisense RNA or a ribozyme.

15. The Human Immunodeficiency Virus of claim 1, wherein the genome of said virus further encodes a gene capable of killing a cell.

16. The Human Immunodeficiency virus of claim 15, wherein said gene encodes Ricin A subunit.

17. The Human Immunodeficiency Virus of claim 1, wherein said virus is a lymphotrophic virus.

18. The Human Immunodeficiency Virus of claim 1, wherein said virus is a macrophage tropic virus.

19. The Human Immunodeficiency Virus of claim 1, 10 or 15, wherein said long terminal repeat sequence further

encodes a mutation which renders said virus replication-incompetent in cells of the nervous system.

20. The Human Immunodeficiency Virus of claim 19, wherein said mutation is a linker substitution mutation.

21. A method of treating a human patient infected with Human Immunodeficiency Virus comprising
first, obtaining a sample of T lymphocytes from the blood of said patient;
second, infecting said T lymphocytes with the Human Immunodeficiency Virus of claim 10 or 15; and,
third, returning said infected T lymphocytes to the blood of said patient.

22. A method of treating a human patient infected with Human Immunodeficiency Virus comprising
first, obtaining a sample of T lymphocytes from the blood of said patient;
second, infecting said T lymphocytes with the Human Immunodeficiency Virus of claim 19; and,
third, returning said infected T lymphocytes to the blood of said patient.

23. A method of treating a human patient infected with Human Immunodeficiency Virus comprising
first, obtaining a sample of monocytes from the blood of said patient;
second, infecting said monocytes with the Human Immunodeficiency Virus of claim 10 or 15; and,
third, returning said infected monocytes to the blood of said patient.

24. A method of treating a human patient infected with Human Immunodeficiency Virus comprising
first, obtaining a sample of monocytes from the blood of said patient;

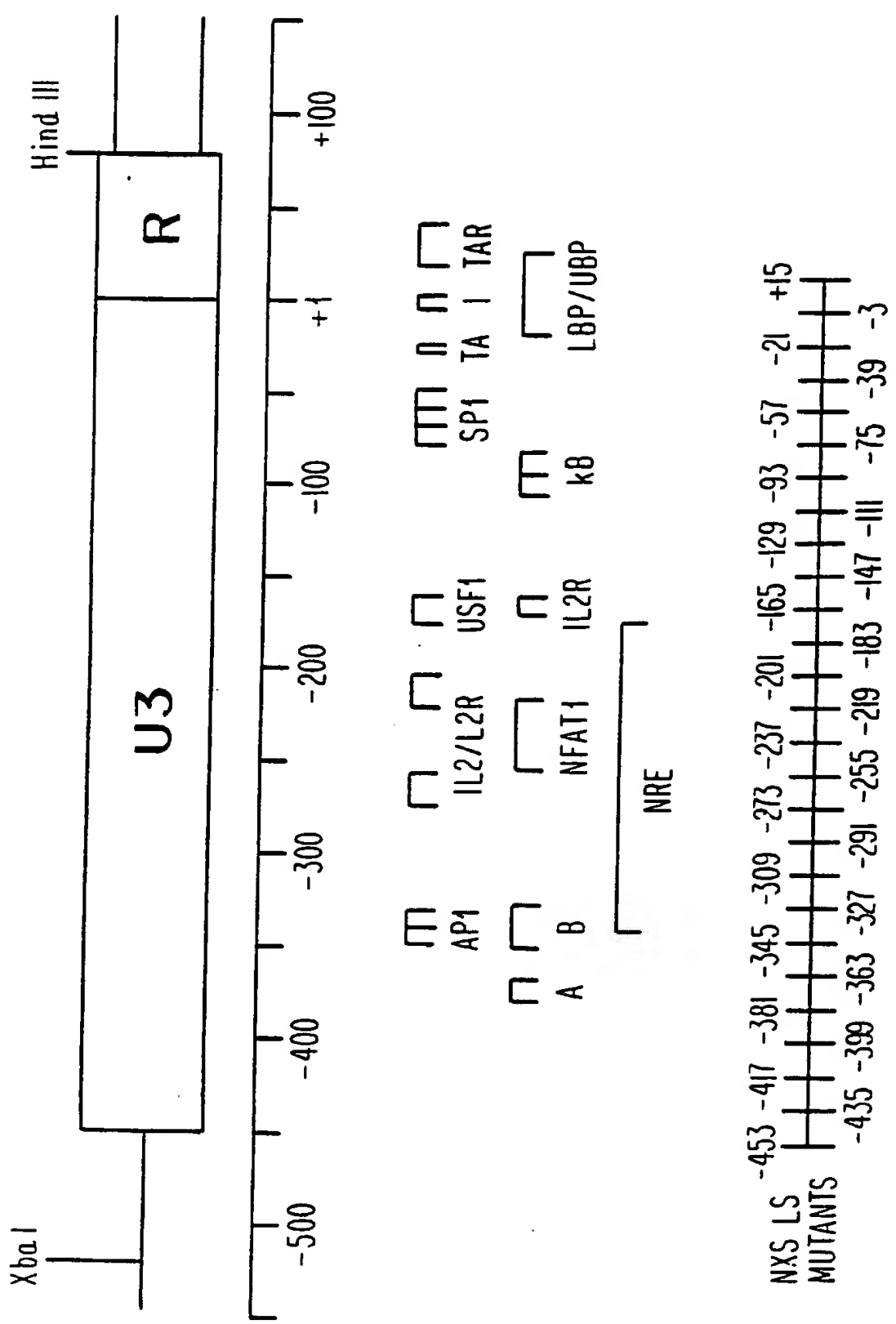
second, infecting said monocytes with the Human Immunodeficiency Virus of claim 19; and,
third, returning said infected monocytes to the blood of said patient.

25. A method of treating a human patient infected with Human Immunodeficiency Virus, said method comprising,
first, obtaining a sample of bone marrow cells from said patient;
second, infecting said cells with the Human Immunodeficiency Virus of claim 10 or 15;
third, returning said infected bone marrow cells to said patient.

26. A method of treating a human patient infected with Human Immunodeficiency Virus, said method comprising,
first, obtaining a sample of bone marrow cells from said patient;
second, infecting said cells with the Human Immunodeficiency Virus of claim 19;
third, returning said infected bone marrow cells to said patient.

27. A method of treating a human patient infected with Human Immunodeficiency Virus, comprising administering the Human Immunodeficiency Virus of claim 10 or 15 directly into the blood stream of said patient, said virus being administered at a dosage of 10^3 to 10^8 $TCID_{50}$ per ml of a pharmaceutically acceptable carrier.

28. A method of treating a human patient infected with Human Immunodeficiency Virus, comprising administering the Human Immunodeficiency Virus of claim 19 directly into the blood stream of said patient, said virus being administered at a dosage of 10^3 to 10^8 $TCID_{50}$ per ml of a pharmaceutically acceptable carrier.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-201
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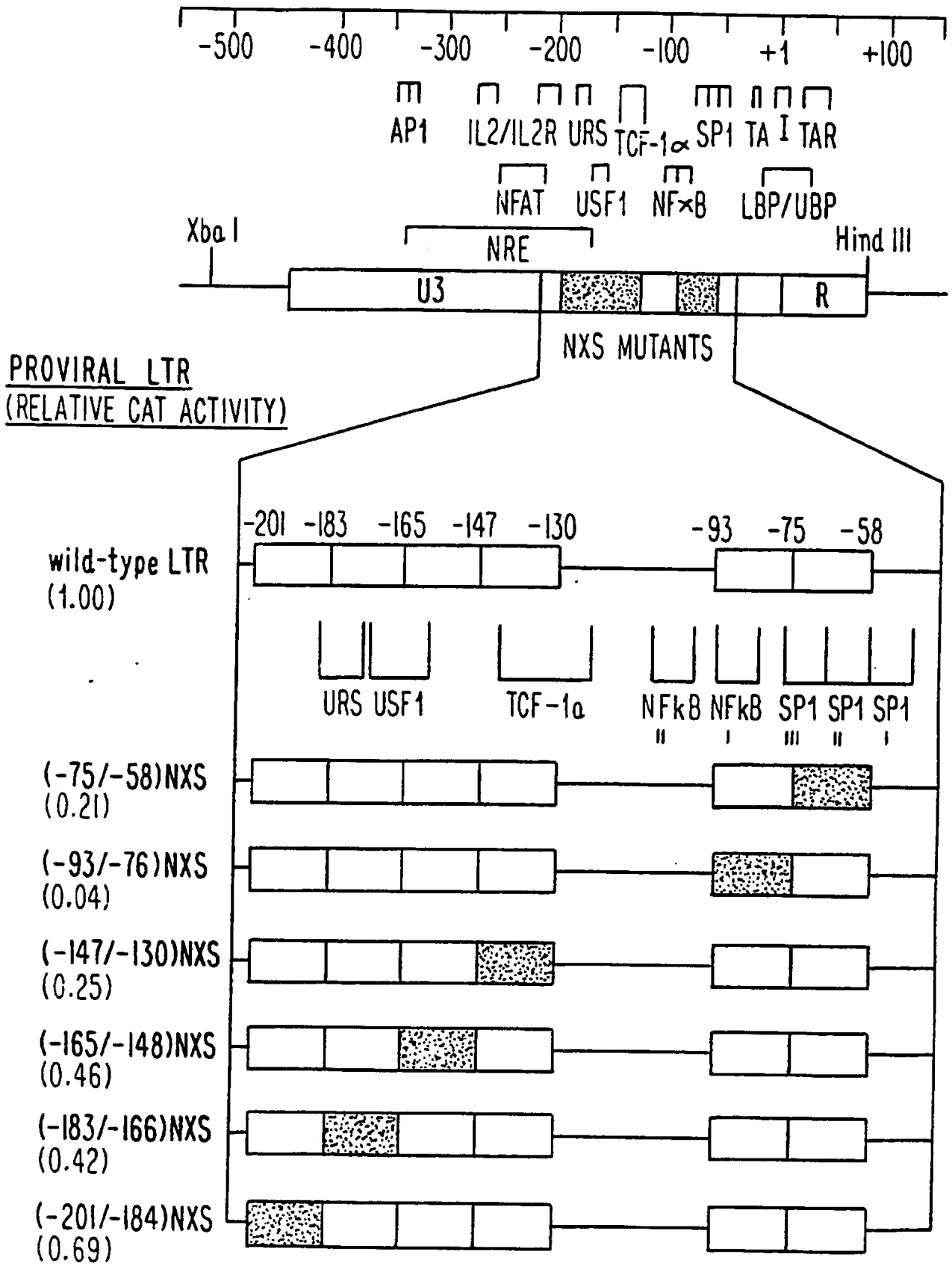
Fig. 1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+1
+100
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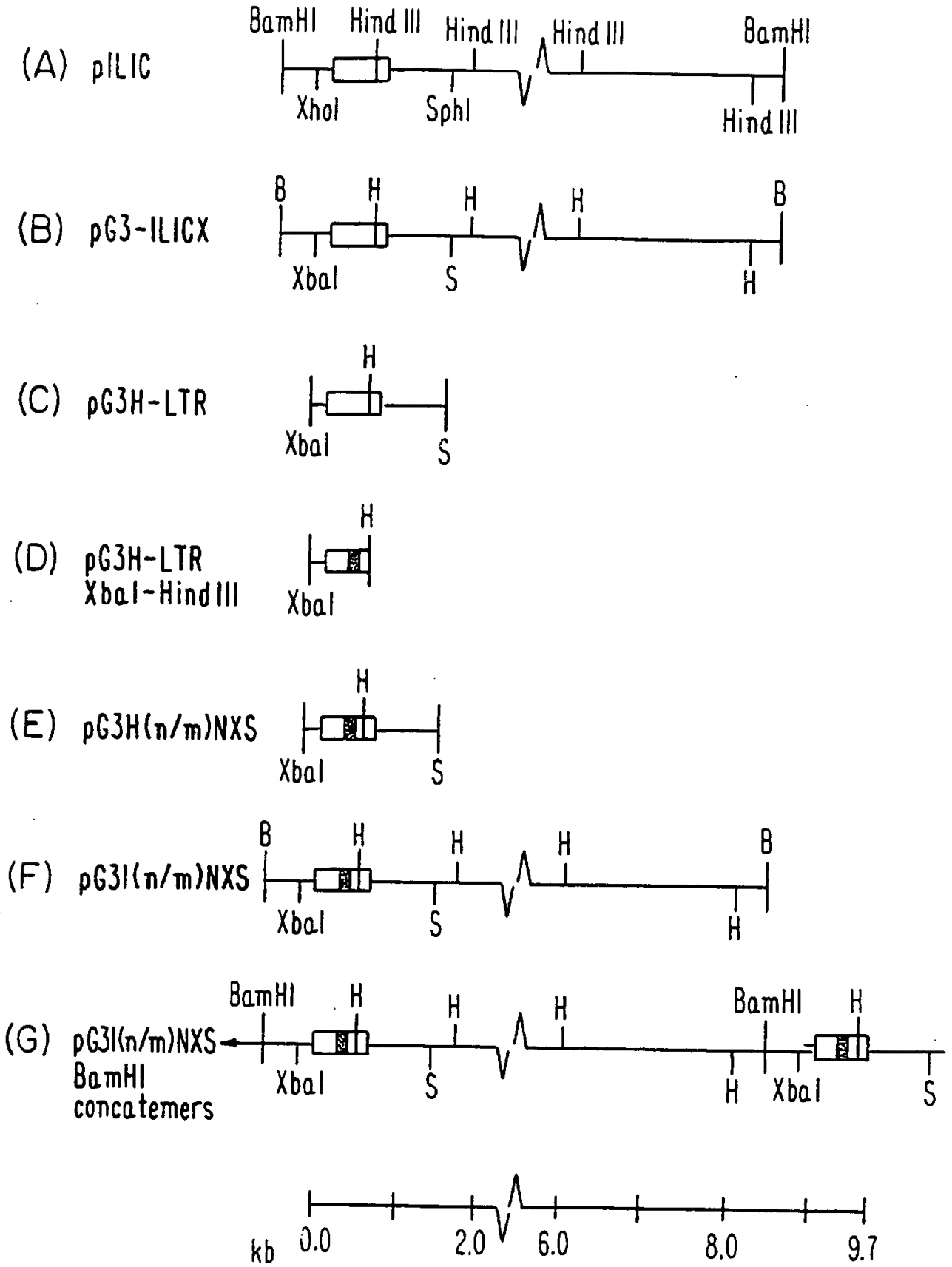
IL2/IL2R
URS
TCF-1α
SP1
TA I
TAR
NFAT
USF1
NFκB
LBP/UBP
Xba I
NRE
Hind III
U3
R
NXS MUTANTS
PROVIRAL LTR
(RELATIVE CAT ACTIVITY)
-201 -183 -165 -147 -130
-93 -75 -58
wild-type LTR
(1.00)
URS USF1
TCF-1α
NFkB NFkB
SP1 SP1 SP1
(-75/-58)NXS
(0.21)
(-93/-76)NXS
(0.04)
(-147/-130)NXS
(0.25)
(-165/-148)NXS
(0.46)
(-183/-166)NXS
(0.42)
(-201/-184)NXS
(0.69)

Fig. 2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BamHI
Hind III
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H
H
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H
H
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Fig. 3

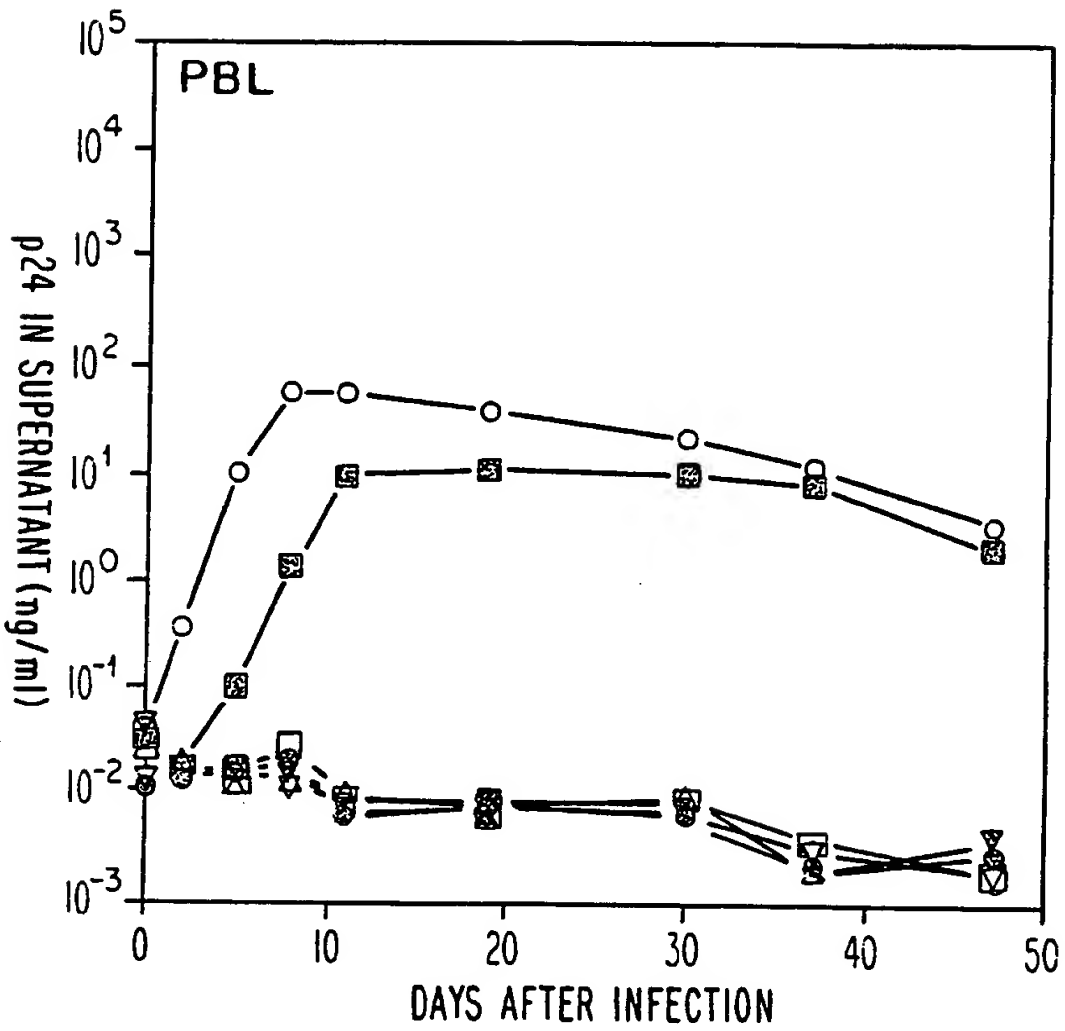
NL4-3(wt)
-75/-58 NXS
-93/-76 NXS
-147/-130 NXS
-165/-148 NXS
-183/-166 NXS
-201/-184 NXS
PBL
p24 IN SUPERNATANT (ng/ml)
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
0
10
20
30
40
50
DAYS AFTER INFECTION

Fig. 4A

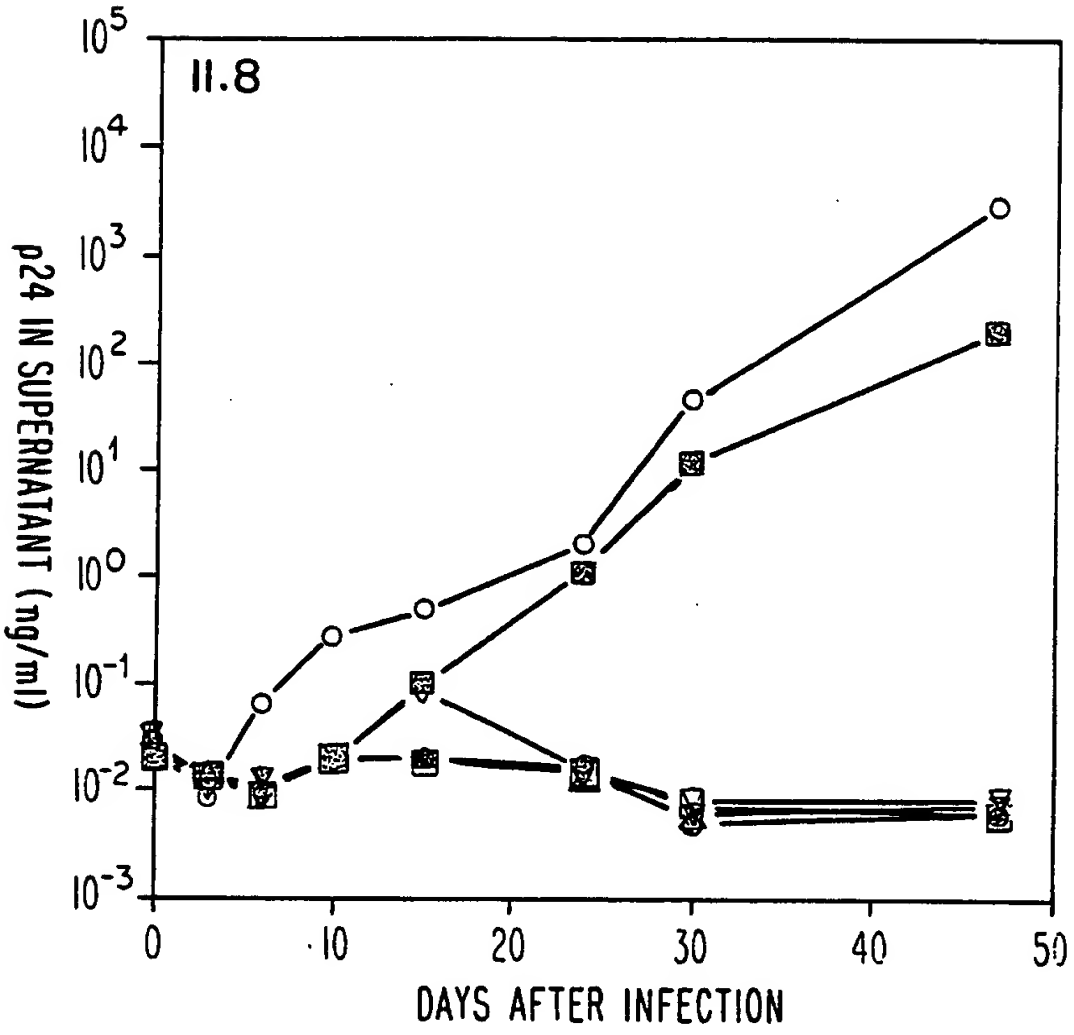
NL4-3(wt)
-75/-58 NXS
-93/-76 NXS
-147/-130 NXS
-165/-148 NXS
-183/-166 NXS
-201/-184 NXS
11.8
p24 IN SUPERNATANT (ng/ml)
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
0
10
20
30
40
50
DAYS AFTER INFECTION

Fig. 4B

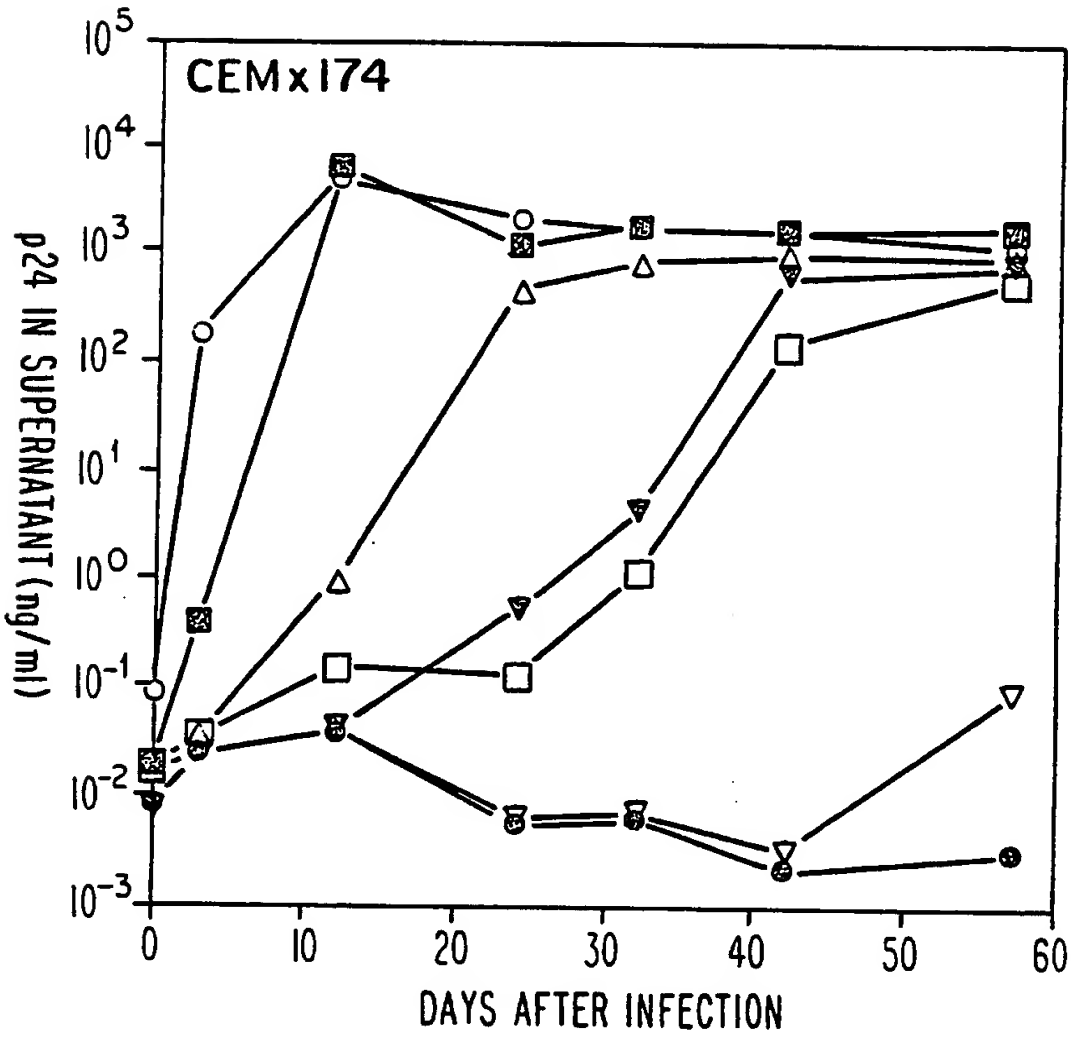
NL4-3(wt)
-75/-58 NXS
-93/-76 NXS
-147/-130 NXS
-165/-148 NXS
-183/-166 NXS
-201/-184 NXS
CEM x 174
p24 IN SUPERNATANT (ng/ml)
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
0
10
20
30
40
50
60
DAYS AFTER INFECTION

Fig. 4C

INTERNATIONAL SEARCH REPORT

Int'l. tional application No.
PCT/US94/00377

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 7/01, 7/04, 15/49; A61K 48/00
US CL :435/320.1, 236; 424/93R, 93A, 93T
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 236; 424/93R, 93A, 93T

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS PREVIEWS, MEDLINE, AIDSLINE, WORLD PATENTS INDEX, CA SEARCH, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Vol. 65, No. 8, issued August 1991, E. K. Ross et al., "Contribution of NF-κB and Sp1 Binding Motifs to the Replicative Capacity of Human Immunodeficiency Virus Type 1: Distinct Patterns of Viral Growth Are Determined by T-Cell Types," pages 4350-4358. See entire article.	1-28
Y	Gene Expression, Vol. 1, No. 1, issued April 1991, S. L. Zeichner et al., "Analysis of the human immunodeficiency virus long terminal repeat by in vitro transcription competition and linker scanning mutagenesis," pages 15-27. See entire article.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 MARCH 1994	MAR 28 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer Jill Warden for JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Int: ional application No.
PCT/US94/00377

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Virology, Vol. 65, No. 5, issued May 1991, S. L. Zeichner et al., "Linker-Scanning Mutational Analysis of the Transcriptional Activity of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat", pages 2436-2444. See entire article.	1-28
Y	Journal of Virology, Vol. 66, No. 4, issued April 1992, S. L. Zeichner et al., "Differentiation-Dependent Human Immunodeficiency Virus Long Terminal Repeat Regulatory Elements Active in Human Teratocarcinoma Cells", pages 2268-2273. See entire article.	1-28
Y	Nature, Vol. 335, issued 29 September 1988, D. Baltimore, "Gene Therapy: Intracellular immunization", pages 395-396. See entire article.	10-15, 21-28
Y	Proc. Natl. Acad. Sci. USA, Vol. 87, issued July 1990, L. Pearson et al., "A transdominant *tat* mutant that inhibits tat-induced gene expression from the human immunodeficiency virus long terminal repeat", pages 5079-5083. See entire article.	10-15, 21-28
Y	J. Exp. Med., Vol. 176, issued October 1992, M. H. Malim et al., "Stable Expression of Transdominant Rev Protein in Human T Cells Inhibits Human Immunodeficiency Virus Replication", pages 1197-1201. See entire article.	10-15, 21-28
Y	Cell, Vol. 59, issued 06 October 1989, D. Trono et al., "HIV-1 Gag Mutants Can Dominantly Interfere with the Replication of the Wild-Type Virus", pages 113-120. See entire article.	10-15, 21-28
Y	Science, Vol. 258, issued 30 October 1992, L. Thompson., "Researchers Test Gene Therapy Against AIDS", page 745. See entire article.	10-16, 21-28
Y	Genes & Development, Vol. 2, issued 1988, C. P. Landel et al., "Lens-specific expression of recombinant ricin induces developmental defects in the eyes of transgenic mice", pages 1168-1178. See entire article.	16